

Correlation of Thiopurine Methyltransferase Activity and 6-Thioguanine Nucleotide Concentration in Han Chinese Patients Treated with Azathioprine 25 to 100 mg: A 1-Year, Single-Center, Prospective Study

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ABSTRACT

Background: Of the enzymes involved in the metabolism of azathioprine, thiopurine methyltransferase (TPMT) is the one characterized by genetic polymorphisms and ethnic variations. There have been several studies of the ethnic variations in phenotype and genotype of TPMT, although few have assessed the possible correlation between TPMT activity and 6-thioguanine nucleotide (6-TGN) concentrations.

Objective: The aim of this study was to examine the relationship between TPMT activity and the steady-state concentration (C_{ss}) of 6-TGN, the primary active metabolite of azathioprine, in red blood cells (RBCs) in Han Chinese patients treated with azathioprine.

Methods: Han Chinese patients aged 18 to 60 years with immunosuppression and normal hepatic and renal function who had been receiving a stable dose (25–100 mg/d) of oral azathioprine as a part of their regular anti-immunosuppression regimen for at least 10 days were recruited for this 1-year, single-center, prospective study. Azathioprine was administered PO QD in the morning, in combination with a stable regimen of other immunosuppressive drugs, for 1 year. At 1 year, blood samples were drawn just before the ingestion of azathioprine. TPMT activity and 6-TGN C_{ss} in RBCs were determined in our laboratory using high-performance liquid chromatography. Adverse drug events were monitored by a patient questionnaire and laboratory testing. Out of the initial cohort, several patients were concurrently enrolled in a subanalysis in which the effect of TPMT polymorphism on the pharmacokinetic prop-

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erties of 6-mercaptopurine, the intermediate metabolite of azathioprine, was examined.

Results: Nineteen patients (14 women, 5 men; mean [SD] age, 41 [9.6] years [range, 22–59 years]; mean [SD] weight, 62 [12] kg) were included in the study; 7 were included in the subanalysis. A significant negative correlation was found between TPMT activity and 6-TGN C_{ss} in RBCs ($r = -0.712$; $P = 0.001$); when the outlier data were removed, no significant correlation was found. Mean (SD) TPMT activity was 12.95 (3.07) nmol/h · mL⁻¹ RBCs and the interindividual CV was 23.68%. Mean (SD) 6-TGN C_{ss} was 42.95 (41.98) ng/8 × 10⁸ RBCs and the interindividual CV was 97.74% ($N = 19$), while the intraindividual CV of 6-TGNs within 8 hours after azathioprine ingestion was between 4.23% and 7.37% ($n = 7$). No significant correlation was found between 6-TGN C_{ss} in RBCs and the dose of azathioprine used. One patient's treatment was discontinued because her white blood cell count decreased to $<4 \times 10^9$ cells/L, indicating myelotoxicity; the $t_{1/2}$ of 6-TGNs in this patient was 5.85 days. Treatment was well tolerated by all other patients.

Conclusion: In this small study, a significant negative correlation was found between TPMT activity and 6-TGN concentration in the RBCs of these Han Chinese patients. However, the correlation was not significant when data from 1 patient with low TPMT activity were excluded. (*Curr Ther Res Clin Exp.* 2006; 67:270–282) Copyright © 2006 Excerpta Medica, Inc.

Key words: thiopurine methyltransferase, azathioprine, 6-thioguanine nucleotides, genetic polymorphism.

INTRODUCTION

Azathioprine has been used extensively to treat immunosuppression in autoimmune diseases and organ transplantation for nearly 40 years.¹ Since 2000, a better understanding of azathioprine metabolism has been gained.^{1,2} Azathioprine is cleaved to 6-mercaptopurine (6-MP) in vivo by glutathione. The 6-MP is then metabolized via 3 competitive routes: (1) by thiopurine methyltransferase (TPMT), resulting in the formation of 6-methyl-mercaptopurine (6-MeMP); (2) by xanthine oxidase (XO), resulting in the formation of the inactive metabolite 6-thiouric acid, which is excreted by the kidneys; and (3) by hypoxanthine-guanine phosphoribosyl transferase (HGPRT), resulting in the formation of 6-thioguanine nucleotides (6-TGNs), which are the primary active metabolites of azathioprine (**Figure 1**).

Of the enzymes involved in the metabolism of azathioprine, TPMT is the one characterized by genetic polymorphisms and ethnic variations. For example, in 1 study,³ TPMT activity displayed a trimodal distribution in a white population: ~89% inherited 2 wild-type alleles with normal (or high) activity; 11% were heterozygous for mutant/wild-type alleles with intermediate activity; and ~0.3% inherited 2 mutant alleles with negligible TPMT activity. In another study in a

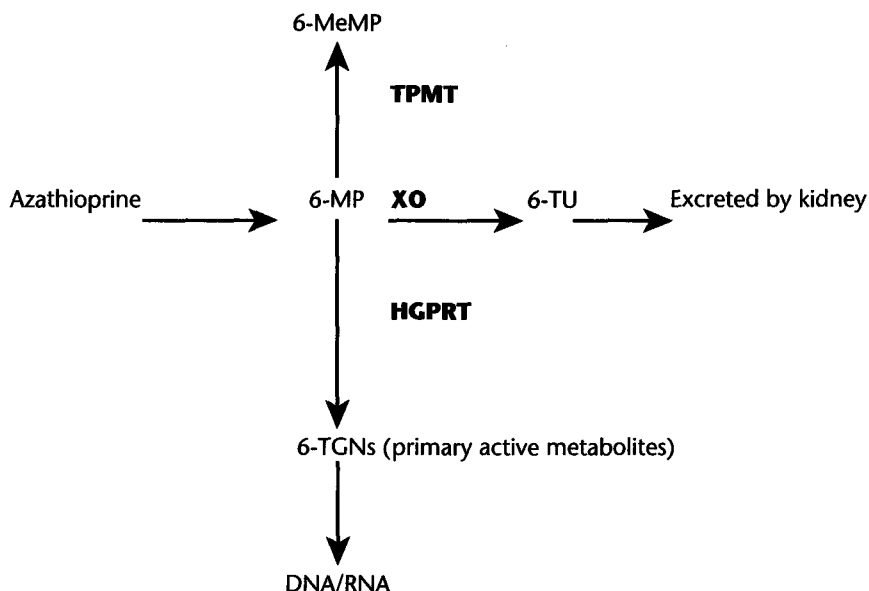


Figure 1. The metabolic pathway of azathioprine. 6-MeMP = 6-methyl-mercaptopurine; TPMT = thiopurine methyltransferase; 6-MP = 6-mercaptopurine; XO = xanthine oxidase; 6-TU = 6-thiouric acid; HGPRT = hypoxanthine-guanine phosphoribosyl transferase; 6-TGNs = 6-thioguanine nucleotides. TPMT, XO, and HGPRT represent the 3 competitive routes by which 6-MP is metabolized.

white population,⁴ the frequency of individuals with a variant TPMT allele was 10.1%, with *TPMT*3A* being the most common mutant allele. However, in a study in 192 Han Chinese patients,⁵ unimodal distribution was found in TPMT activity, with the mean (SD) activity being 12.12 (3.27) U/mL red blood cells (RBCs). The frequency of mutant alleles was 4.7% (9/192); the most common mutant allele was *TPMT*3C*.⁵

Several studies have assessed the ethnic variations in the phenotype and genotype of TPMT,⁶⁻⁹ although few studies have assessed both TPMT activity and 6-TGN concentrations.³ The correlation between TPMT activity and the 6-TGN concentration in RBCs remains controversial.¹⁰⁻¹⁴ A search of the literature did not find any such studies in the Han Chinese population (Chinese Biomedical Literature Database search [in Chinese]; key terms: *azathioprine*, *thiopurine methyltransferase*, *6-thioguanine*, and *polymorphism*; years: 1978–2005). Furthermore, in patients with high TPMT activity, 6-MP is readily metabolized into 6-MeMP by TPMT, decreasing the production of 6-TGNs. For these reasons, we examined the relationship between TPMT activity and steady-state 6-TGN concentration (C_{ss}) in the RBCs of Han Chinese patients.

PATIENTS AND METHODS

Study Design

This 1-year, single-center, prospective study was conducted at Peking Union Medical College Hospital (Beijing, China). Han Chinese patients aged 18 to 60 years with immunosuppression and normal hepatic and renal function who had been receiving azathioprine as a part of their regular anti-immunosuppression regimen at a stable dose (25–100 mg/d) for at least 10 days were recruited for this study. According to a previous study,¹⁵ 6-TGNs reach C_{ss} 7 to 10 days after the initiation of azathioprine therapy. Patients receiving concomitant medications that might affect the metabolism of azathioprine, such as XO inhibitors (eg, sulfasalazine or allopurinol) or liver microsomal enzyme inducers or inhibitors,³ were excluded. Patients who had received a blood transfusion within 4 months before the study were also excluded. Written informed consent was obtained from each patient before enrollment, and the study was approved by the institutional review board of our institution.

Azathioprine was administered PO QD in the morning, in combination with a stable regimen of other immunosuppressive drugs, for 1 year. At 1 year, a blood sample was collected from each patient in 5-mL heparinized tubes just before the ingestion of azathioprine. For a subanalysis in which the effect of TPMT polymorphism on the pharmacokinetic properties of 6-MP, the intermediate metabolite of azathioprine, was examined, blood samples were also drawn at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 7, and 8 hours after azathioprine administration in some patients.

Tolerability was assessed by adverse events elicited using a patient questionnaire and routine laboratory tests (blood [including liver and kidney function studies] and urine tests) administered once a week for the first month and once a month for the remainder of the study. Treatment with azathioprine was to be discontinued in any patient whose white blood cell (WBC) count decreased to $<4 \times 10^9$ cells/L, indicating myelotoxicity, and blood testing was to be done weekly thereafter until the WBC count returned to normal ($4.5\text{--}11.0 \times 10^9$ cells/L). The $t_{1/2}$ of 6-TGN in RBCs was to be determined in the patients who discontinued azathioprine treatment.

Blood Sample Preparation

Blood samples were processed according to the method used by Weinshilboum et al.¹⁶ Briefly, samples were centrifuged at 300g for 10 minutes at 4°C, and the plasma was discarded. The RBCs were washed twice in isotonic saline, and then 2 mL of isotonic saline was added to the pellets. After the cells were resuspended, hematocrit and RBC count were measured using an automated cell counter (Celltac MEK-6108k, Nihon-Kohdon, Tokyo, Japan). Then, 0.5 mL of the resuspended RBCs was added to 2 mL of ice-cold water, resulting in the lysis of the RBCs. The RBC lysates were centrifuged (RC5C, Sorvall Instruments, DuPont, Wilmington, Delaware) at 13,000g for 15 minutes at 4°C, and the supernatant was used for the TPMT assay. The remaining resuspended

RBCs were used for the 6-TGN assay. Samples were frozen at approximately -80°C until analyzed.

Thiopurine Methyltransferase Activity Assay

TPMT activity in RBCs was determined using a slightly modified high-performance liquid chromatography (HPLC) assay, as described previously.^{16,17} Metronidazole was used as the internal standard in this assay.¹⁸ The retention time of metronidazole is similar to that of azathioprine; no interference peaks were found in the retention time of metronidazole. The stability of metronidazole was verified using HPLC. Based on the TPMT-catalyzed conversion of 6-MP to 6-MeMP using S-adenosyl-L-methionine (SAM) as the methyl donor, 100 μL of the RBC lysate was incubated with 100 μL of potassium phosphate buffer (0.15 mol/L; pH 7.5), 25 μL of 6-MP (24 mmol/L), 20 μL of SAM (112.5 $\mu\text{mol/L}$), 10 μL of dithiothreitol (5.6 mmol/L), and 10 μL of allopurinol (450 $\mu\text{mol/L}$) for 1 hour at 37°C . The reaction was stopped by adding 50 μL of hydrochloride (1 mol/L), and then metronidazole was added as the internal standard. 6-MeMP was extracted into dichloromethane-2-propanol (80:20 v/v). After being centrifuged, the organic phase was evaporated with nitrogen until dry, and the residue was dissolved in 120 μL of mobile phase. An injection volume of 50 μL was selected. 6-MeMP was assayed using a UV detector ($\lambda = 290\text{ nm}$) with a reverse-phase C18 column (Shim-pack, CLC-ODS, 6 mm \times 150 mm, 5- μm internal diameter, Shimadzu, Kyoto, Japan). The mobile phase consisted of methanol, water, and triethylamine in a 24.0:75.6:0.4 ratio (v/v), and the flow rate was maintained at 1.0 mL/min. A standard curve ranging from 50 to 2000 ng/mL was selected, and linearity was confirmed by linear regression ($r = 0.9996$). The CVs were 4.29% for the interday and intraday assays, and the recoveries were between 94.9% and 103.2% in control samples. In a blood sample from one of the authors (B.Z.), which was used as a control sample, the intraday and interday CVs of TPMT activity were 3.29% and 3.25%, respectively, throughout the course of the study.

TPMT activity was expressed as nanomoles of 6-MP formed per hour per milliliter of packed RBCs ($\text{nmol/h} \cdot \text{mL}^{-1}$).

6-Thioguanine Nucleotide Concentration Assay

We developed an HPLC method to determine the 6-TGN C_{ss} in RBCs with slight modifications based on those by Dervieux and Bouliou.¹⁹ 6-MP was introduced into this method as the internal standard. The retention time of 6-MP was determined to be similar to that of azathioprine; no interference peaks were found. The stability of 6-MP was verified using HPLC.

6-TGN C_{ss} was determined using a UV detector ($\lambda = 345\text{ nm}$) with the reverse-phase C18 column. The mobile phase consisted of methanol, water, and triethylamine in a 10.0:89.8:0.2 ratio (v/v). An injection volume of 50 μL was selected, and the flow rate was maintained at 1.0 mL/min. Protein was removed from 200 μL of RBCs using 70% perchloric acid with dithiothreitol. The nucleotides were hydrolyzed to their base 6-thioguanine (6-TG) by heating the supernatant for 1 hour

at 100°C. A standard curve ranging from 5 to 750 ng/mL was selected, and linearity was confirmed by linear regression ($r = 0.9997$). The intraday and interday CVs obtained in checked samples ranged from 2.27% to 6.27%, and the recoveries in the samples ranged from 96.9% to 101.7%.

6-TGN C_{ss} was expressed as nanograms of 6-TG per 8×10^8 RBCs (ng/ 8×10^8 RBCs).

Statistical Analysis

The correlation between variables was determined with the Spearman non-parametric rank correlation analysis using SPSS version 10.0 (SPSS Inc., Chicago, Illinois). $P < 0.01$ and $\alpha = 0.01$ were considered statistically significant. No power analysis was done to determine sample size a priori.

RESULTS

Study Population

Nineteen patients (14 women, 5 men; mean [SD] age, 41 [9.6] years [range, 22–59 years]; mean [SD] weight, 62 [12] kg) were enrolled in this study (Table I). Seven of the 19 patients were enrolled in the subanalysis.

Thiopurine Methyltransferase Activity and 6-Thioguanine Nucleotide Concentrations in Red Blood Cells

TPMT activity in the 19 patients ranged from 3.50 to 17.87 nmol/h \cdot mL⁻¹ RBCs. Mean (SD) TPMT activity was 12.95 (3.07) nmol/h \cdot mL⁻¹ RBCs, and the interindividual CV was 23.68% (Table II).

6-TGN C_{ss} in the RBCs of the 19 patients ranged from 7.56 to 187.73 ng/ 8×10^8 RBCs. Mean (SD) 6-TGN C_{ss} was 42.95 (41.98) ng/ 8×10^8 RBCs, and the interindividual CV was 97.74% (Table II). A 24.8-fold interindividual variation in 6-TGN C_{ss} , which was larger than that in TPMT activity (5.11-fold), was observed.

Correlation Analysis

No significant correlation was found between the dose of azathioprine and 6-TGN C_{ss} in RBCs. A significant negative correlation was found between TPMT activity and 6-TGN C_{ss} in RBCs ($r = -0.712$; $P = 0.001$). However, when the outlier data (6-TGN C_{ss} , 187.73 ng/ 8×10^8 RBCs; TPMT activity, 3.5 nmol/h \cdot mL⁻¹ RBCs) were removed, no significant correlation was observed. A scatterplot of TPMT activity and 6-TGN C_{ss} values is shown in Figure 2.

Pharmacokinetic Properties of 6-Thioguanine Nucleotides

In the subanalysis, little intraindividual variation was found in 6-TGN C_{ss} within 8 hours after azathioprine administration, and the CV was between 4.23% and 7.37%. The concentration–time curves of 6-TGNs in the RBCs in these 7 patients after PO azathioprine 100 mg are shown in Figure 3.

Table I. Baseline demographic and clinical characteristics of 19 Han Chinese patients with immunosuppression.

Patient No.	Sex	Age, y	Weight, kg	Azathioprine Dose, mg/d	Diagnosis
1	F	32	41	50	SLE
2	F	30	61	25	SLE
3	F	22	64	100	LN
4	F	28	55	50	CTD
5	F	40	85	50	SLE
6	F	47	62	100	CTD
7	F	54	55	50	SS
8	F	37	45	100	LN
9	F	50	61	100	AIH
10	F	42	75	50	SS
11	F	43	57	100	SLE
12	F	30	62	50	SLE
13	F	35	42	100	KT
14	M	43	70	50	KT
15	M	46	65	100	KT
16	M	50	78	100	KT
17	M	46	75	100	KT
18	F	38	51	100	KT
19	M	59	75	100	KT
All patients					
Mean (SD)		41 (9.6)	62 (12.0)	78 (28.0)	—
CV, %		23.41	19.35	35.90	—

F = female; SLE = systemic lupus erythematosus; LN = lupus nephritis; CTD = connective tissue disease; SS = Sjögren's syndrome; AIH = autoimmune hepatitis; KT = kidney transplantation; M = male.

The WBC count in 1 patient who was not included in the subanalysis decreased from 4.5×10^9 cells/L to 3.8×10^9 cells/L. Following the study's protocol, azathioprine treatment was then discontinued and blood samples were collected weekly after discontinuation. Four weeks after discontinuation of azathioprine, the patient's WBC count had returned to normal (5.8×10^9 cells/L). The $t_{1/2}$ of 6-TGN in the RBCs of this patient was 5.85 days (**Figure 4**).

Tolerability

No deaths or serious adverse events occurred. The only abnormal laboratory test result was the low WBC count (3.8×10^9 cells/L) in 1 patient. No other adverse events were reported in the remaining 18 patients.

Table II. Thiopurine methyltransferase (TPMT) activity and 6-thioguanine nucleotide (6-TGN) concentration in red blood cells (RBCs) from 19 Han Chinese patients with immunosuppression treated with azathioprine 25 to 100 mg for 1 year.

Patient No.	TPMT Activity, nmol/h · mL ⁻¹ RBCs	6-TGN Concentration, ng/8 × 10 ⁸ RBCs
1	13.65	16.14
2	14.51	7.56
3	14.14	31.62
4	12.13	13.97
5	8.80	31.24
6	16.36	45.17
7	12.39	26.33
8	17.87	9.84
9	13.72	65.16
10	3.50	187.73
11	14.39	40.88
12	13.66	20.78
13	14.12	84.79
14	14.16	43.69
15	10.01	89.16
16	12.27	31.65
17	13.76	32.33
18	14.96	14.47
19	11.62	23.55
All patients		
Mean (SD)	12.95 (3.07)	42.95 (41.98)
CV, %	23.68	97.74

DISCUSSION

TPMT, which is an important enzyme in the metabolism of purine drugs (eg, azathioprine, 6-MP, and 6-TG), is characterized by genetic polymorphisms and ethnic variations. In this study, a significant negative correlation between TPMT activity and 6-TGN C_{ss} in RBCs ($r = -0.712$; $P = 0.001$) was found in 19 patients, which was consistent with the results of previous studies.¹¹⁻¹³

One patient in this study had the highest 6-TGN concentration and the lowest TPMT activity in the study. When the data for that patient were removed, the correlation between TPMT activity and 6-TGN C_{ss} in RBCs was no longer significant. In our opinion, this was a rare case and further studies (eg, genotype analysis and pharmacokinetic studies) should be done. This information was only determined after the physician had already recommended discontinuation of azathioprine based on the opinion that the patient would not bene-

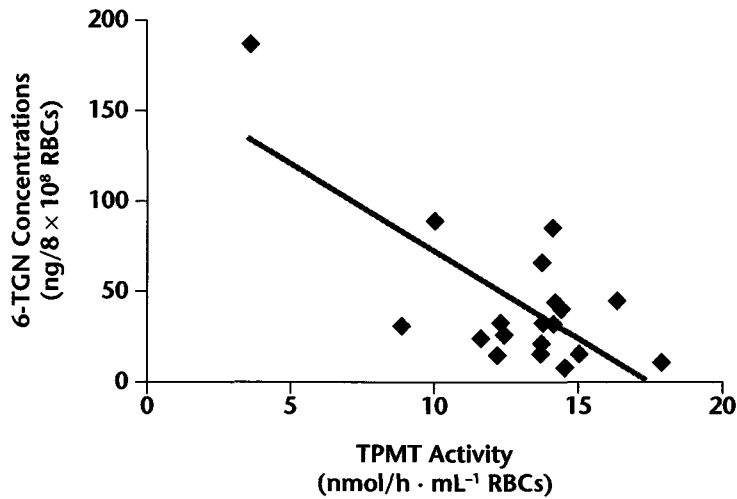


Figure 2. Thiopurine methyltransferase (TPMT) activity and steady-state concentration of 6-thioguanine nucleotides (6-TGNs) in red blood cells (RBCs) in Han Chinese patients with immunosuppression treated with azathioprine 25 to 100 mg for 1 year (N = 19) ($r = -0.712$; $P = 0.001$).

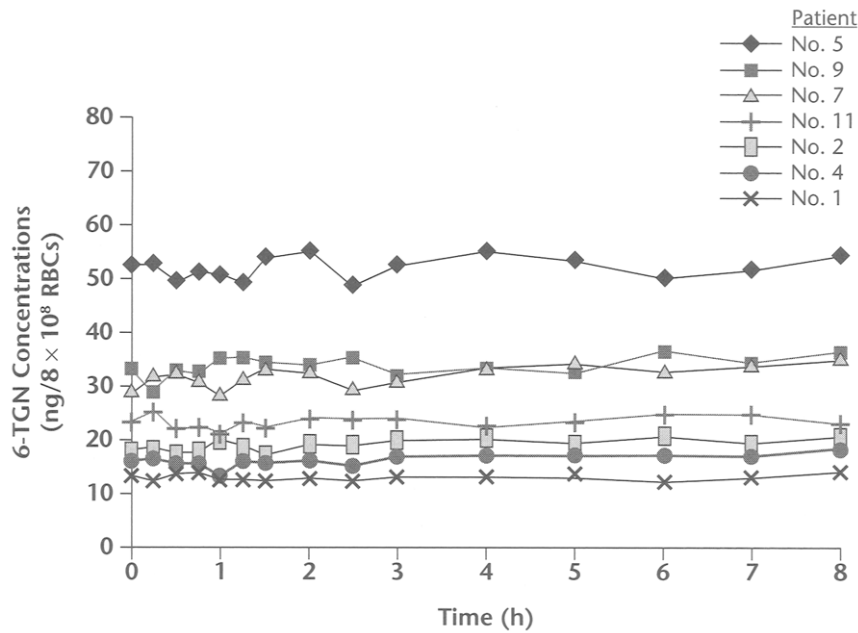


Figure 3. Concentration-time curve of 6-thioguanine nucleotides (6-TGNs) in red blood cells (RBCs) in Han Chinese patients with immunosuppression treated with azathioprine 100 mg for 1 year (n = 7).

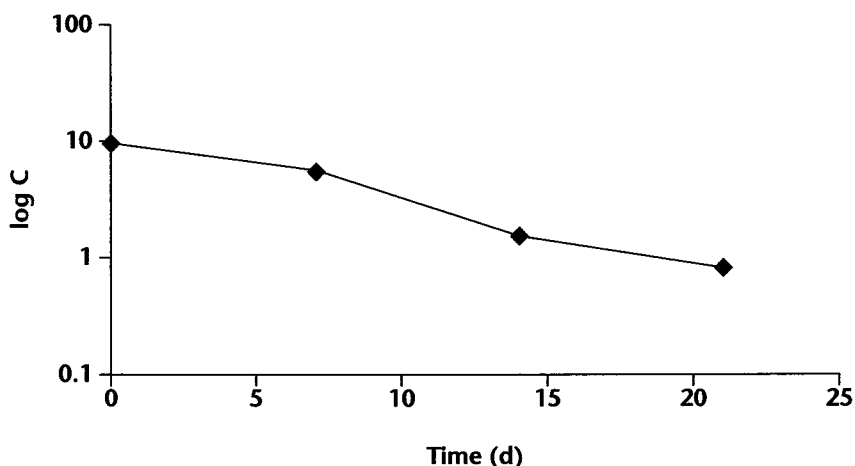


Figure 4. Concentration-time curve of 6-thioguanine nucleotides (6-TGNs) in red blood cells in the 1 Han Chinese patient whose azathioprine treatment was discontinued due to a low (3.8×10^9 cells/L) white blood cell count. log C = logarithm of 6-TGN concentration.

fit from the treatment. The response to the high 6-TGN C_{ss} might have been associated with increased toxicity if this patient had continued treatment with azathioprine.

As for the pharmacokinetic properties of 6-TGNs in RBCs, little intraindividual variation in 6-TGN C_{ss} ($\leq 7.37\%$) was found within 8 hours of azathioprine administration, suggesting that if it becomes necessary to monitor 6-TGN concentrations in clinical practice, then blood samples can be collected within 8 hours of azathioprine ingestion.

The $t_{1/2}$ of 6-TGN in RBCs was 5.85 days in the patient in whom a low WBC count developed. Lennard et al²⁰ reported that the $t_{1/2}$ of 6-TGN in RBCs was ~3 to 13 days in kidney transplant recipients. The long $t_{1/2}$ of 6-TGNs in our patient was consistent with the slow onset of action of azathioprine and the duration of recovery from myelotoxicity found by Lennard et al. Our patient's WBC count decreased from 4.5×10^9 cells/L to 3.8×10^9 cells/L on day 15 after the initiation of azathioprine treatment; however, the 6-TGN concentration in that patient was only 9.84 ng/ 8×10^8 RBCs. Myelotoxicity has been reported in a heart transplant recipient whose 6-TGN C_{ss} was 369.68 ng/ 8×10^8 RBCs²¹ and in a patient with juvenile human leukocyte antigen B27-associated spondylarthritis whose 6-TGN C_{ss} was 400.28 ng/ 8×10^8 RBCs.²² Given the differences in the concentration of 6-TGN in RBCs found in those studies, it appears unlikely that the case of myelotoxicity in our study was associated with 6-TGNs.

Our study had several limitations. First, the number of patients in this study was small ($N = 19$), and only 1 patient had low TPMT activity, which was not unexpected given the low frequency of a mutant allele in the Han Chinese pop-

ulation (4.7%).⁴ Second, the patients were clinically heterogeneous (12 had autoimmune diseases and 7 were kidney transplant recipients). Finally, genotype analysis was not performed in this study. Factors other than TPMT activity might have affected the 6-TGN C_{ss} , such as variation in XO or HGPRT activities and/or bioavailability of azathioprine.

In clinical practice, azathioprine toxicity is monitored using routine blood tests. A WBC count of $<4 \times 10^9$ cells/L indicates myelotoxicity, and recovery from myelotoxicity might be quite difficult, making the initial dose of azathioprine important. Our study found a negative correlation between TPMT activity and 6-TGN C_{ss} in these Han Chinese patients treated with azathioprine. Because patients with low or absent RBC TPMT activity may be at increased risk for complications due to elevated 6-TGN C_{ss} , it may be useful to measure RBC TPMT activity before beginning azathioprine treatment. In patients with low or absent activity, the azathioprine dose might need to be reduced at the beginning of treatment to prevent serious adverse events, and such patients should be carefully monitored. In patients with high TPMT activity, the 6-TGN C_{ss} might be less than that considered therapeutic, which could necessitate increasing the dose of azathioprine to achieve a therapeutic outcome.

CONCLUSION

In this small study, a significant negative correlation was found between TPMT activity and 6-TGN C_{ss} in RBCs in these Han Chinese patients receiving a stable dose of azathioprine as part of a regular treatment regimen for immunosuppression. However, the correlation was not significant when the outlier data were excluded.

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